Research Paper

Effect of CO, NOx and SO2 on ROS production, photosynthesis and ascorbate–glutathione pathway to induce Fragaria × annasa as a hyperaccumulator

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A B S T R A C T

A study was conducted to determine the effect of carbon monoxide (CO), nitrooxide (NOx) and sulfur dioxide (SO2) on ROS production, photosynthesis and ascorbate–glutathione pathway in strawberry plants. The results showed that both singlet oxygen (O2–1) and hydrogen peroxide (H2O2) content increased in CO, NOx, and SO2 treated strawberry leaves. A drastic reduction of primary metabolism of plants (photosynthesis), with the closure of stoma, resulted in a reduction of protein, carbohydrate and sucrose content due to production of reactive oxygen species (ROS) under prolonged exposure of gas stress. The resulting antioxidant enzymes were increased under a low dose of gas stress, whereas they were decreased due to a high dose of gas stress. Our results indicate that increased ROS may act as a signal to induce defense responses to CO, NOx and SO2 gas stress. The increased level of antioxidant enzymes plays a significant role in plant protection due to which strawberry plants can be used as a hyperaccumulator to maintain environmental pollution, however, the defense capacity cannot sufficiently alleviate oxidative damage under prolonged exposure of CO, NOx, and SO2 stress.

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Introduction

Environmental pollution from industrial effluents and other organic pollutants has become a major environmental and human concern worldwide [1,2]. The major hazardous gases released from industries are abundant in number, among which carbon monoxide (CO) is a piousness gas to mammals, and has many similar qualities and effects to those of carbon dioxide (CO2), except for its flammability at high concentration [3]. As an important signaling molecule in physiological activity, CO has a similar toxic effect to that of cyanide, the inhibitory effect of a higher ratio of CO to O2 (above 4:1) on cytochrome c oxidase in the mitochondrial electron transport chain at the cellular level or the effect on the detoxification enzyme P-450 in the monooxygenase system and multiprotein complex proteins in chloroplasts. CO can have important signaling roles as well as toxic effects, but this depends upon the amount produced and context, as plants also contain a variety of heme moiety-containing proteins (catalase, peroxidase, cytochromes, etc.), and produce a number of important, biliverdin-related tetrapyrrrole pigments, such as phycocyanobilin [4] and phytochromes [5].

On the other hand nitrogen oxides (NOx) are produced by combustion processes and NOx is usually >90% nitric oxide (NO), with the balance being composed of nitrogen dioxide (NO2). In the atmosphere, chemical reactions convert NO to NO2, which can further react to produce O3. While NOx emission rates vary from plant to plant, largely according to the design of the plant and the characteristics of the fuel, an uncontrolled emission rate of 1.0 b mmBtu−1 is typical for coal-fired generators. NOx is a free radical gas which transfers electrons across biological membranes [6].

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase; APX, ascorbate peroxidase; H2O2, hydrogen peroxide; O2–1, singlet oxygen; CO, carbon monoxide; NOx, nitrooxide; SO2, sulfur dioxide, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Rubisco, ribulose-1,5-bisphosphate carboxylase oxygenase; SS, larger subunit; S, smaller subunit

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In general the electron acceptor is oxygen and the product of the electron transfer reaction is superoxide. The biological function of NOx is therefore the generation of reactive oxygen species (ROS) [6]. The physiological generation of ROS can occur as a byproduct of other biological reactions. ROS generated as a byproduct occurs within mitochondria, peroxisomes and other cellular elements [7].

Sulfur dioxide (SO2) is one of the most common harmful air pollutants. The phytotoxicity of SO2 depends on its concentration and on the duration of exposure [1] and is influenced by sulfur status in plants [8]. The low dose of sulfur even can be useful to plants since sulfur is important for plants, and can help to alleviate other nutrient deficiencies in plants, such as that of Fe [9]. Sulfur is also a structural component of amino acids, proteins, vitamins and chlorophyll [8,10,11]. Sulfur enhances the development of nodules and nitrogen fixation, and also affects carbohydrate metabolism. However, exposure to high doses can lead to leaf chlorosis and necrosis, growth inhibition and plant death [12]. The concentration of SO2 in the atmosphere has increased in many countries during the past few decades. It is estimated that parts of global plants exposed to SO2 have prompted widespread attempts to evaluate defense responses.

The overall toxicity of these hazardous gases CO, NOx and SO2 is derived from toxic molecular species such as those from the combustion of CO2 to CO, NOx to NO2 as well as O3, sulphite (SO32-) and bisulphite (HSO3-). The detoxification reaction of these compounds further leads to the production of reactive oxygen species, such as hydroxyl radical (OH•) and hydrogen peroxide. Increased production of ROS can attack bio-macromolecules and results in oxidative damage to nucleic acids, proteins and lipids [13,14]. However, plants can scavenge excess ROS by invoking the antioxidant defense system to avoid oxidative damage. Many studies have shown that some antioxidant enzymes are involved in plant response under various abiotic stresses. The induction of antioxidant enzymes is thought to be a protective reaction of plants againstabiotic stress, but the exact defense mechanism is not clear.

Strawberry plants are the richest grown plants in greenhouses which are often fertilized by direct CO2 enrichments fertilizers for heating. The CO2 boilers/direct heating systems used in greenhouses however, often lead to incomplete combustion which results in formation of hazardous gases such as carbon monoxide (CO), nitric oxide (NO), nitric dioxide (NO2) as well as O3, sulphite (SO32-) and bisulphite (HSO3-). The homogenates were centrifuged at 5000 g for 3 min at 4 °C in 100 mM Tris buffer (pH 7.8) and incubated at 25 °C for 1 h. After incubation the mixture was added with 0.5 ml of 17 mM alpha aminobenzene sulphonic acid and 0.5 ml of 7 mM alpha naphthylamine solution and was incubated again for 20 min at 25 °C and absorbance was recorded at 530 nm.

**Determination of H2O2 and O2-**

H2O2 concentration was measured calorimetrically as described by Lin and Kao [15] using titanium sulfate. H2O2 concentration was calculated using the extinction coefficient 0.28 mM−1 cm−1 and was expressed as nmol g−1 tissue fresh weight. For O2− determination fresh tissue was extracted in phosphate buffer (pH 7.5) and centrifuged at 10,000g for 5 min. The resultant extract was added to 1 ml of 10 mM NH2OH·HCl and phosphate buffer (pH 7.8) and incubated at 25 °C for 1 h. After incubation the mixture was added with 0.5 ml of 17 mM alpha aminobenzene sulphonic acid and 0.5 ml of 7 mM alpha naphthylamine solution and was incubated again for 20 min at 25 °C and absorbance was recorded at 530 nm.

**Measurement of net photosynthetic activity and Fv/Fm ratio**

Photosynthetic rate was measured using a portable photosynthesis measurement system (LI-6400XT, LI-COR, Inc., Lincoln, NE). Chlorophyll fluorescence (Fv/Fm) was measured by using a chlorophyll fluorescence meter (PAM 2000, Heinz Walz GmbH, Effeltrich, Germany). The leaves were adapted to dark conditions for 30 min before the measurement. The maximum fluorescence (Fm) and minimum fluorescence (F0) were determined by applying a saturating light pulse (20 kHz) of 1100 µmol m−2 s−1 PPF for 3 µs. The maximum PS II quantum yield (Fv/Fm) was calculated as Fv/Fm=(Fm−F0)/Fm for 24 h at different concentrations of CO, NOx and SO2.

**Photosynthetic pigments and stomatal observation**

The content of chlorophyll and carotenoid was estimated by the method of Hiscox and Israclstam [16]. The fresh leaves were collected in glass vials, to which 10 ml dimethyl sulfoxide (DMSO) was added, after which samples were kept in an oven at 65 °C for complete leaching of pigments for 1 h. Optical density was recorded at 480, 645, 520 and 663 nm. The content of total chlorophyll and carotenoid was calculated using the formulae given by Arnon [17]. For stomatal observation, thin layers of leaf tissues were carefully cut and were laid on glass slides, covered with cover slips by adding few drops of water, and were observed under a light microscope (Leica DM4000 M) at 40 × magnification.

**Rubisco determination by SDS-PAGE**

Leaf tissues were homogenized at 4 °C in 100 mM Tris buffer (pH 7.5) containing 5 mM of DTT, 2 mM iodoacetate and 5% (v/v) glycerol at a leaf; buffer ratio of 1:5–10 (g:ml). For this extraction, a buffer without sodium or potassium ion was recommended for SDS-PAGE analysis because those cations reduce the solubility of DS (dodecyl sulfate). Before centrifugation, a TritonX100 (25%, v/v) was added to a portion of leaf homogenate to make a final concentration of 0.1% (v/v). An addition of TritonX100 was effective for the extraction of Rubisco bound to the membrane fraction [18]. The homogenates were centrifuged at 5000g for 3 min at 4 °C. A lithium DS solution (25% w/v) and 2-mercaptoethanol were added to the supernatant fluid to a final concentration of 1.0%
Total carbohydrate and sucrose content

The carbohydrate content was determined by anthrone method [19]. One hundred milligrams of dried leaf tissue were added to a boiling tube. The tissues were hydrolyzed in a boiling tube by keeping them in a water bath for 3 h with 5 ml of 2.5 N-HCl, and were then cooled at room temperature. The solution was then neutralized with solid sodium carbonate until effervescence ceased, and samples were then centrifuged. After centrifugation 1 ml of supernatant had added solid sodium carbonate until effervescence ceased, and samples were cooled at room temperature. The solution was then neutralized with them in a water bath for 3 h with 5 ml of 2.5 N-HCl, and were then centrifuged at 15,000 g for 10 min. One unit of enzyme was expressed as the amount necessary to decompose 1 μmol of ascorbate in 1 min.

Antioxidant enzyme assays

Superoxide dismutase (SOD) activity was determined by the method of Dhinda et al. [20] with minor modifications. Fresh tissues (200 mg) were extracted in phosphate buffer (pH 7.3) and were then centrifuged at 15,000g. SOD activity in the supernatant was assayed for its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) by reading the absorbance at 560 nm. RuBisCO content was determined as the amount of enzyme necessary to decompose 1 μmol of ascorbate according to Nokano et al. [23]. One hundred milligrams of fresh tissue were extracted in 100 mM K-phosphate (pH 7.0), and were then centrifuged at 15,000g for 10 min. One unit of enzyme was expressed as the amount necessary to decompose 1 μmol of ascorbate in 1 min.

Statistical analysis

A completely randomized design was used with three biological replicates for five treatments. An individual Student’s t test was employed to compare the means of separate replicates by using software SAS (version 9.1, USA).

Results and discussion

CO, NOx and SO2 induced ROS generation

Reactive oxygen species generation was observed by determination of hydrogen peroxide (H2O2) and singlet oxygen (O2–1). Strawberry leaves which were exposed to CO, NOx, and SO2 gases exhibited high concentration of H2O2 and O2–1 (Fig. 1A and B). The concentration of H2O2 and O2–1 was observed highest by more than 90 and 80% respectively under high dose of CO, NOx, and SO2 compared to control.

In plant cells, ROS are unavoidable by-products of aerobic metabolism. Under normal growth conditions, the amount of ROS is modest, and cells experience only mild oxidative stress, whereas, many stresses enhance ROS production [13,24,25]. The results of our study clearly show that CO, NOx, and SO2 triggered the rapid induction of O2–1 and H2O2 generation in strawberry leaves. This enhanced production of ROS under stress can pose a threat to cells and can also act as a signal to activate stress response pathways such as the ascorbate–glutathione cycle [13,26].

Photic parameters

The net photosynthetic rate in plants exposed under low doses of hazardous gases (CO, NOx, and SO2) showed a slight reduction of net photosynthesis, however, at the medium dose of gas stress the net photosynthetic rate decreased (Fig. 2A) and the reduction of photosynthesis was observed to be highest in plants under high doses of CO, NOx, and SO2.

Similarly, the ratio between Fv/Fm under CO, NOx and SO2 treatment was not different between control plants, and those exposed to a low dose of gas stress (Fig. 2B). After a medium dose of gas stress, the ratio between Fv/Fm decreased, and the lowest ratio was observed under a high dose of gas stress by 13%, 24% and 11%, respectively, when compared to the control.

We also observed that photosynthetic pigments (total chlorophyll and carotenoid) under CO, NOx, and SO2 were reduced slightly under a low dose of gas stress, whereas under a medium dose of hazardous gas stress the photosynthetic pigments reduced significantly and the highest reduction was observed in plants under a high dose of gas stress, reducing total chlorophyll by 54%, 60% and 59% (Fig. 3A) and carotenoid content by 66%, 80% and 70% (Fig. 3B) compared to control.

The present results show that photosynthetic parameters such as net-photosynthesis, Fv/Fm ratio and photosynthetic pigments (chlorophyll and carotenoid) were reduced under CO, NOx, and SO2, which represents that there might be a transfer of lesser electrons and lesser energy investment in the biochemical machinery of strawberry. The reduction of net photosynthesis and ratio between Fv/Fm has been also observed under salt stress in green algae [27] and in Vigna radiata under mineral deficiency [24,25]. The decline in net photosynthesis and Fv/Fm under hazardous gas stress might be due to the inactivation of reaction centers of

(w/v) and 1% (v/v), respectively. This preparation was immediately treated at 100 °C for 1 min, and was then stored at −30 °C until the analysis of SDS-PAGE. The samples containing 2–10 μg RuBisCO were loaded onto 12% polyacrylamide gel. After electrophoresis, the gels were stained in 0.25% (w/v) CBB-R. The stained bands corresponding to larger and smaller subunits of RuBisCO were cut out of the gels with a razor blade and were eluted in 1–2.5 ml of formamide in a stoppered amber test tube at 50 °C for 5 h with shaking. The absorbance of the resultant solution was read at 595 nm with a spectrophotometer. RuBisCO content was determined by using the standard curve calculated from the absorbance of a known amount of purified RuBisCO.
photosystems which receive an initial amount of light energy and cannot be exploited efficiently due to the presence of oxidative stress. The ineffective energy exploitation [28,29] leads to an extreme increase of dissipated energy, and therefore, decreases photosynthesis. In addition to this the loss of carotenoids might be due to the loss of chlorophyll, because carotenoids absorb light energy for use in photosynthesis, and they protect chlorophyll from photo-damage. Such a decrease in chlorophyll content may also be attributed to a reduction of hazardous gases for the formation of precursor molecules, δ-aminolevulinic acid and protochlorophyllide [30].

**Observation of stomata under microscope**

Microscopic observations (40 × ) have shown that stomata did not show any change in behavior in plants under low dose of CO, NOx, or SO2 treatment (Figs. 4 and 5). With increased doses of gas exposure the stomata gradually closed and guard/subsidiary cells were damaged compared to those of the control, which was found to be exaggerated at high dose of gas exposed after 24 h.

As we know, stomata play an important regulatory role in leaf physiological processes, as the primary pathway for the exchange of gases between internal leaf surfaces and the atmosphere [31]. The closing of stomata as a result of exposure under hazardous gas has been reported in several species such as *Vicia faba* [32] and *Pisum sativum* [31] under SO2 stress. The closure of stomata under high concentrations of CO, NOx and SO2 demonstrated that certain key enzymes involved in stomatal metabolism might have been affected, which are also involved in several other metabolic activities in plants such as malate formation and metabolism in epidermis, and turgor pressure of guard cells. The destructive damage of guard cells and density might also occur due to defoliation of leaves during growth conditions of strawberry, and indeed, due to high temperatures under hazardous gas conditions [33], which might have resulted in the induction of palisade and sponge parenchyma cell length and thickness.
RuBisCO contents

Fig. 4 shows that the RuBisCO content determined by SDS-PAGE, the RuBisCO content in plants under low dose CO, NO\(_x\), or SO\(_2\) showed great change in expression, and statistically significant results, as generated by a spectrophotometer, have shown a huge change even early stages of hazardous gas treatment. With increasing hazardous gas doses the reduction of RuBisCO content was observed to be highest in plants exposed to low doses of gas stress when compared to that of the control.

The changes in RuBisCO content in response to various abiotic stresses has been reported in many species, such as in *Trifolium alexandrinum* [34] under salt stress conditions. The maintenance of a higher RuBisCO content in plants has been correlated with a higher rate of photosynthesis in certain genotypes, and a lower content of RuBisCO is correlated with lower rates of photosynthesis under abiotic stresses [35]. In the present study, the reduction of RuBisCO content under hazardous gas stress (CO, NO\(_x\), and SO\(_2\)) coincides with a reduction of photosynthesis (Fig. 2). In recent studies we observed the proteomic data of *Brassica napus* under Fe-deficiency to reduce most proteins classified as photosynthetic precursors, including RuBisCO content [10]. The loss of RuBisCO protein might be due to the progressive depletion of biochemical pathways associated with signal transduction and gene regulation, and in particular, might be involved in protein synthesis [36,37], and also might be associated with an excessive production of ROS which leads to incorrect folding or assembly of proteins, and consequent protein degradation [38].

**Fig. 4.** Representative images of stomata, as affected by low, medium and high doses of CO, NO\(_x\), and SO\(_2\). Thin layer of leaf outer covering were peeled off carefully and laid on a glass slide, covered with a cover slip, and were observed under a light microscope (Leica CME) at 40 x magnification.
Carbohydrate and sugar content

The total carbohydrate in plants under low dose of CO, NOx or SO2 was reduced compared to control (Fig. 6A) and more reduction was observed in plants under medium dose of hazardous gas stress by 54%, 81% and 72% respectively. The reduction was observed to be highest under high dose of CO, NOx and SO2, by 65%, 87% and 70% respectively.

Sucrose content in plants under low dose of CO, NOx and SO2 showed a slight reduction, which was greater (and significant) in medium dose plants (Fig. 6B), however, a high dose of CO, NOx and SO2 reduced the sucrose content by 23%, 48% and 65%, respectively, when compared to control.

The decrease in total carbohydrates and sucrose content of damaged leaves probably corresponded with photosynthetic inhibition, or with the stimulation of a lower respiratory rate. Higher starch accumulation in damaged leaves may have resulted both in the higher resistance of their photosynthetic apparatus [39] and low starch export from the mesophyll. The negative effect of hazardous gases on carbon metabolism is a result of their possible interaction with the reactive center of ribulose bisphosphate carboxylase [40].

CO\textsubscript{2}, NO\textsubscript{x} and SO\textsubscript{2}-induced antioxidative defense response

We analyze the evolution of stress responses in strawberry plants exposed to low, medium and high doses of CO, NO\textsubscript{x} or SO\textsubscript{2} for 24 h. The treatment evokes physiological changes in the form of ascorbate glutathione cycle. There was a stark contrast between the responses of all antioxidative enzymes examined in this study. CO, NO\textsubscript{x} and SO\textsubscript{2} increased SOD, CAT, GR and APX, activity at low and medium doses of gas stress conditions (Fig. 7A–D), while at higher doses, SOD activity was decreased by 55%, 55.1% and 78%, catalase activity was decreased by 81%, 89% and 86%, GR activity was decreased by 69%, 30% and 47% and APX activity was decreased by 32%, 62% and 25% compared with control.

In this study, the activities of antioxidative enzymes (APX, SOD, CAT and GR) were found to be increased at low and medium doses of CO, NO\textsubscript{x} and SO\textsubscript{2} stress, whereas they were significantly decreased at high doses of gas stress. Several reviews have been published on different aspects of ascorbate and glutathione, ranging from their biosynthesis [41,42], to their roles in the transport system [43] and stress defense [44] in plants. It has been generally established that glutathione and ascorbate play a prominent role in non-enzymatic mechanisms to prevent the oxidation of cellular compounds [44], and are involved in the induction of enzymes and gene expression. SOD catalyzes the dismutation reaction of O\textsubscript{2}\textsuperscript{-1} and H\textsubscript{2}O\textsubscript{2} for the initial period of stress conditions, as induction of these enzymes was observed in our study, whereas, after prolonged exposure the SOD could not dismutate these ROS into H\textsubscript{2}O\textsubscript{2} and H\textsubscript{2}O, which resulted in the reduction of enzyme activities of ascorbate glutathione cycle. Glutathione is involved as a substrate for glutathione peroxidase and is therefore necessary for the removal of lipid peroxidases, because GR reduces the oxidized glutathione, which is generated by glutathione peroxidase [42,45]. The observed increase in GR at initial period of stress resulted in the detoxification of oxidative damage, while a high dose of CO, NO\textsubscript{x} and SO\textsubscript{2} resulted in a disturbance in ascorbate glutathione pathways. CAT and APX are the primary H\textsubscript{2}O\textsubscript{2} scavenging enzymes in plants. In this study, CO, NO\textsubscript{x} and SO\textsubscript{2} pronouncedly increased APX activity and CAT activity in the initial period of stress, which indicates that APX and CAT are efficient enzymes for detoxifying ROS arising from hazardous gas treatments. Moreover the increased activity of these enzymes can contribute to resistance and enhancement since they participate in many other physiological processes in plant defense reactions.

Conclusions

In conclusion, this is a systematic study which provided a specific insight into the changes in ROS production, photosynthesis...
Fig. 7. Changes in antioxidant enzyme activities (A) SOD (B) CAT (C) GR and (D) APX as affected by low, medium and high dose of CO, NO, and SO2. Vertical bars indicate ± SE by means with n = 3. Means are denoted by different letters are significantly different at p < 0.05 according to Tukey's studentized range test.

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